THE RELATION BETWEEN CELL INTEGRITY AND BACTERIAL LUMINESCENCE

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In 1902 Macfadyen found that luminous bacteria exposed to liquid air would glow again on rewarming, but gave no light on rewarming if thoroughly ground at the temperature of liquid air. Harvey (1915) tried to obtain luminescence from cytolyzed and dried crushed bacteria, but even though the possibility of oxidation of the bacterial luciferin by molecular oxygen was precluded (a precaution Macfadyen did not take) by grinding the bacteria in the desiccated form or by cytolyzing them with toluol, ether, water, and other agents in the absence of oxygen, no light was obtained on moistening the ground bacteria, or aerating the cytolysate. Intact, dead, desiccated bacteria luminesced for a short time when moistened, even after prolonged extraction with fat solvents such as ether, absolute alcohol, toluol, benzine, and chloroform. These agents acting on moist or suspended bacteria, however, invariably and quickly destroyed permanently the ability to luminesce.

The demonstration of a given reaction in cellular extracts has always been a much more difficult problem in the case of bacteria than in other cells, due to the relatively greater importance of the cell structure. According to Quastel (1926) catalytic dehydrogenations, particularly, are very closely associated with the surface of the bacterial cell.

The demonstration of luminescence apart from cell structure is an even more difficult problem since it is necessary to extract both the catalyst, luciferase, and its specific substrate, luciferin. The normally close association between the two is very likely disturbed, due, conceivably, to differences in solubility between the two, to the instability of one or both apart from the normal structure of the cell, or to the oxidation of the luciferin by oxidants other than oxygen.

New methods have been applied in a continued investigation of this problem, namely, the attempt to demonstrate luminous substances from luminous bacteria destroyed in various ways. Three species were used: a fresh-water form, *Vibrio phosphorescens*, and two marine

forms from Woods Hole, one unidentified, and the other, *Achromobacter fischeri*, a very brilliant variety. Four general methods, with numerous modifications, were employed for the destruction of the bacteria: (*a*) cytolysis by fat solvents, (*b*) cytolysis with hypotonic solutions (not applicable, of course, to the fresh-water form), (*c*) mechanical grinding, and (*d*) sonic radiation.

Throughout these experiments care was taken to prevent oxidation of the luciferin, and, hence, they were carried out anaerobically. After destruction of the bacteria, the suspensions were aerated in the dark and observations on the presence or absence of luminescence made with completely dark-adapted eyes.

Experiments with Fat Solvents

For this series of experiments and those involving osmotic cytolysis a double trap vessel illustrated in Fig. 1 was found convenient for the anoxic mixing of cytolytic agents with the suspension. The agent was put in one arm of the vessel (*B*), the suspension in the other (*A*), and thoroughly deaerated with a stream of hydrogen purified by passage over red-hot platinized asbestos. The two were then mixed by tilting the vessel. On aerating, no return of luminescence was observed with either chloroform or caprylic alcohol as the cytolytic agent.

A modification of this technique, in which deaeration was accomplished with sodium hydrosulfite, yielded the same results.

These confirm Harvey's (1915) results: suspensions deaerated by evacuating and then cytolyzed with toluol, ether, chloroform or carbon tetrachloride showed no luminescence on re-aerating.

Experiments with the Osmolytic Technique

The cytolytic agent in this series is diluted sea water. A volume of distilled or tap water found, in air, to cause rapid darkening of a given volume of a dense sea-water bacterial suspension is pipetted into one arm of the vessel (*A*), and the suspension into the other (*B*). The rest of the experiment is run as in the above series. Although repeated many times, luminescence has never been obtained from bacteria "cytolyzed" by hypotonic solutions in the absence of oxygen.

Darkening of bacterial suspensions by hypotonic solutions is perhaps not a true indication of complete cytolysis of the bacteria. Although the suspensions do become clear and foamy, due to the release of cellular contents almost immediately after mixing with distilled water, the bacteria remain viable for hours after complete darkening, as shown by inoculating culture media with portions of the suspension

at intervals after complete darkening.[1] Excellent growth will occur after 3½ or more hours exposure to distilled water. Even though true cytolysis by this method is a very slow process, the copious release of cellular contents justifies its application.

It is possible that, despite the most rigorous exclusion of oxygen, the bacterial luciferin, during release from the cell, becomes oxidized (by the more positive systems in the cell) without giving light. Harvey

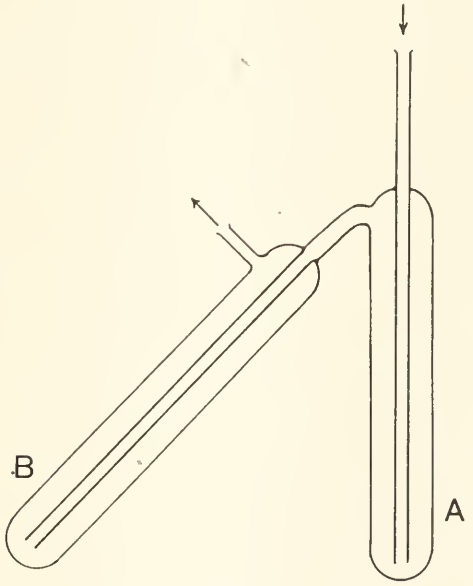

FIG. 1.

(1927) has shown that *Cypridina* luciferin can undergo non-luminescent (and reversible) oxidations not involving molecular oxygen. Strong reducing agents should prevent such oxidations, but the presence of finely divided Pt in the cytolyzing mixture, which, together with the hydrogen used for deaerating, gives a reducing intensity approaching that at a hydrogen electrode, does not alter the results. The same is true if the osmotic cytolysis is allowed to take place in the presence of a

[1] The darkening of bacterial suspensions by hypotonic solutions is doubtless at least partly an osmotic phenomenon, but not entirely, for a volume of tap or distilled water which causes rapid darkening of a given volume of suspension will do so only if added at once. If slowly poured in or added in three or four installments, even closely spaced, the time for darkening is greatly extended, the suspension retaining some luminescence for hours. Another recent and pertinent observation is that the destruction of the capacity for luminescence by hypotonic solutions is very much more rapid under anaerobic conditions than in aerobic. The difference is far too large to be ascribed merely to a difference in the rates of penetration of the water in the presence and absence of oxygen.

small but adequate mount of neutralized sodium hydrosulfite. *Cypridina* oxyluciferin, which gives no light with luciferase, can be reduced by hydrosulfite to luciferin, which does give light with luciferase.

Another possible cause of failure to obtain luminescence from substances liberated from bacteria is that the photogenic substances become diluted or separated, even when liberated into small volumes of liquid, whereas, in the cell, they are quite concentrated and closely associated. The attempt was made in the following experiments to minimize this dispersion by concentrating the substances on adsorbing surfaces. The procedure was exactly that described above for the osmolytic technique, except that an adsorbent, either $Fe(OH)_3$, kaolin, or Norit A was added to the distilled water. For the use of $Fe(OH)_3$ the bacteria had to be suspended in isotonic sucrose to prevent the too rapid precipitation of the sol by the salts of sea water on mixing. In no case was luminescence observed on aeration.

Repetition of these experiments but with the addition of finely divided Pt to both tubes to provide reducing action (together with the H_2) gave the same results.

In some experiments the adsorbents were replaced by fresh-water luminous bacteria from 4–6-day-old cultures which had ceased luminescing, or other fresh-water (non-luminous) forms. But the presence of normal bacterial surfaces instead of those of the colloids did not affect the results.

Attempts were also made to reduce oxyluciferin, liberated from cells by aerobic cytolysis. A thick mass of bacteria (*A. fischeri*), washed and collected by centrifuging, was suspended in water, in aqueous colloidal $Fe(OH)_3$ or in aqueous kaolin suspensions, in air. Some time after complete darkening of the suspension (due to hypotonicity) it was again centrifuged, the supernatant fluid poured off and the thick gelatinous mass at the bottom of the tube quickly dried in vacuo over $CaCl_2$. The dried powder was suspended in water and, in the absence of oxygen, exposed to the action of colloidal $Pt + H_2$. This (again assuming a fundamental similarity between bacterial and *Cypridina* luciferin) should have reduced the oxyluciferin formed during its (aerobic) release from the cell to luciferin. However, no light appeared on aeration in any case.

Autolytic Method

A dense mass of *A. fischeri* was kept at 38° C. for 3 days in order that autolysis might occur. This material, after reduction with $Pt + H_2$ or with hydrosulfite, gave no luminescence on aeration. The autolysate, which should have contained large quantities of bacterial

luciferase (and oxyluciferin), when mixed with bacterial luciferin, i.e. with what would be analogous to a *Cypridina* luciferin preparation (bacteria boiled and cooled in a hydrogen atmosphere), gave no light. Attempts to demonstrate luciferase in ground bacteria, desiccated marine bacteria resuspended in water or adsorption extracts, described in the preceding paragraph, with the above luciferin preparation also proved negative. Likewise, "cross" experiments with *Cypridina* luciferin and luciferase also failed.

Extracts of *Cypridina* which have become dark due to the oxidation of all the luciferin will, when electrolyzed, luminesce in the vicinity of the cathode, due to cathodal reduction (Harvey, 1923). Various extracts of the luminous bacteria, similarly treated, did not luminesce.

Mechanical Destruction of Bacteria

This was accomplished with a device consisting of a ground-glass rod revolving in a close-fitting glass tube whose inner surface in contact with the rod is also ground.[2] (Ten Broeck, 1931; Glaser and Coria, 1935.) The rod was fitted to the shaft of a small electric motor. In order to grind in the absence of oxygen, the grinder and motor were hermetically enclosed in a casement of brass and glass. The glass part consisted of a test tube sealed with deKhotinsky cement into a circular opening in the brass case containing the motor. This glass tube contained the grinding parts, thus permitting observation. Wires to the motor and gas-inlet and -outlet tubes were fitted to the brass case through a rubber stopper, so that the apparatus could be exhausted with a vacuum pump or hydrogen passed through it while grinding was taking place.

A small quantity of thick, bright suspensions was carefully pipetted into the bottom of the grinding vessel without getting bacteria on the walls of the vessel. The bacteria were ground either in an atmosphere of hydrogen or in a vacuum at a high rate of speed for one to two hours, after which air was admitted in the dark and observations made. The temperature never rose above 28° C. If grinding was thorough, no light appeared on aerating. If, before grinding was begun, finely divided Pt was added to the suspension, and the experiment performed in a H_2 atmosphere, the results were also the same, no light appearing on aeration.

Experiments with Sonic Vibration

A number of experiments in which destruction of the bacteria was accomplished by means of a powerful magnetostriction oscillator of

[2] The author is grateful to Dr. R. W. Glaser of Rockefeller Institute, Princeton, for a set of the glass parts, after which others were modelled.

9,000 cycles were performed.[3] Sonic radiation from the apparatus used is known to break apart bacteria into fragments (Chambers and Gaines, 1932) and will extinguish the luminescence of bacterial suspensions in air in 13 to 20 minutes. The suspension was contained in a glass vessel fitted over the vibrating nickel-alloy tube, and deaerated by a stream of nitrogen first passed over heated copper to remove traces of oxygen. The suspension was completely darkened by the removal of dissolved oxygen before the generator was turned on, but for some reason the suspension became slightly luminous again on even momentary irradiation, indicating the presence of a small amount of oxygen. Although this was washed out by the continued stream of N_2, there was almost always a reappearance of luminescence on irradiating. Bacteria, irradiated in the presence of this small concentration of oxygen for 13–20 minutes (the time required depending on the concentration of bacteria) showed no luminescence on blowing air through their suspensions. In only two experiments were we successful in completely deaerating the suspensions (as shown by darkness of the suspensions on momentarily turning on the oscillator). These, also, did not luminesce on being aerated at the end of the run.

The work of Shoup (1929, 1933, 1934) and of Taylor (1932, 1934) has shown that when respiration is inhibited by a variety of methods, e.g. KCN, CO, excessive dinitrophenol, lowered oxygen tension, luminescence is not affected until respiration is reduced to about 40–50 per cent of the normal, when the intensity drops off. Whether the failure of respiration and of luminescence is due to some common factor or whether the failure of respiration bears some causal relation to that of luminescence cannot be readily determined. It is easy to show that the respiration of "cytolyzed" bacteria is greatly affected. As determined by a modification of the dimming-time method their respiration is reduced to far below the 40–50 per cent level, often lower than 10 per cent. It seems that the dehydrogenases are the affected systems, rather than the oxidase-cytochrome system, for the rate of reduction of methylene blue (Thunberg technique) is greatly lowered by osmotic cytolysis while the Nadi test for indophenol oxidase is, if anything, intensified.

Discussion

Even a cursory review of the literature shows that bacterial luminescence is far from unique among biological oxidative reactions in being dependent upon cellular structures and having catalysts not

[3] I am most grateful to Dr. Leslie A. Chambers and the Johnson Foundation for Medical Physics for the use of the oscillator and assistance in its operation.

extractable in the soluble form. Some of the oxidative reactions of blood cells are inhibited or destroyed by osmotic hemolysis, and it is particularly true that the dehydrogenations by bacteria disappear with cell destruction (Quastel and Woolridge, 1927, 1928; Stephenson, 1928; Young, 1929), although some of the dehydrogenations are less dependent on normal structure than others. Other interesting observations are those of Gozony and Suranyi (1925) and of Shwartzman (1926), who found that lysis with phage greatly reduced the rate of methylene blue reduction by bacteria. Clifton (1933) found that lysis with phage causes less negative reduction potentials in suspensions of *Staphylococcus aureus*. I have found that cytolysis (by hypotonic solutions, cytolytic agents, and autolysis) produces similar effects on luminous bacteria. The oxidative reactions observed by Neill and his coworkers (1924–1928) in detritus-free extracts of *Pneumococcus* are all very likely due to the presence of non-enzymic catalysts such as certain intracellular reversibly oxidizable pigments.

Whether luciferase can be classed with the dehydrogenases is, of course, debatable. One fundamental similarity is shown, however, in that luciferin (LH_2) is oxidized to oxyluciferin (L) by a dehydrogenation catalyzed by luciferase. The energy derived from this oxidation is then transferred to the luciferase, which luminesces (Harvey, 1927). *Cypridina* luciferin, however, differs from other hydrogen-donators in being autoxidized in the presence of oxygen, and readily oxidized by certain oxidizing agents, e.g. ferricyanide and quinone, but without the production of light. Hence, for luminescence, the reaction is highly specific.

Although bacterial luminescence and *Cypridina* luminescence are both dependent on molecular oxygen, the inability to demonstrate the luciferin-luciferase reaction or to obtain any luminescence from injured or disintegrated bacterial cells, even under the most favorable conditions, forces us to the conclusion that luminescence in these forms is very decidedly bound up in an intimate manner with structural conditions, presumably unaltered surfaces in or on the cell.

Summary

Three species of luminous bacteria were injured or destroyed by a variety of modifications of four general methods: (*a*) cytolysis with fat solvents, (*b*) osmotic cytolysis, (*c*) mechanical grinding, and (*d*) intense sonic vibration. Although all experiments were performed under conditions which would have prevented the oxidation of the bacterial luciferin and which were, in general, favorable for bioluminescence, it was not possible in any case to demonstrate the luciferin-luciferase

reaction or obtain luminescence from bacteria whose structure had been materially altered. The conclusion is drawn that bioluminescence, like many other bacterial oxidative phenomena, is closely associated with cellular structure. Respiration and reducing activity were shown also to be greatly affected.

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